A STUDY OF THE JELLY ENVELOPES SURROUNDING THE EGG OF THE AMPHIBIAN, XENOPUS LAEVIS[1]

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The greatest interest in the oviducal jelly material deposited around amphibian eggs has centered on its possible significance in the processes of meiosis (Humphries, 1961) and fertilization (Hughes, 1957; Kambara, 1953; Katagiri, 1963b, 1966; McLaughlin, 1967; Nadamitsu, 1957; Rugh, 1935, 1951; Shaver and Barch, 1960; Shivers and Metz, 1962; Subtelny and Bradt, 1961; Tchou and Wang, 1956). It is generally accepted that the jelly is necessary for successful fertilization of amphibian eggs; however, the particular properties of the jelly which make its presence essential to fertilization are largely unknown. Kambara (1953, p. 84) has suggested that the jelly " . . . plays the role of a mechanical foothold which enables the sperm to penetrate the eggs." In contrast, Katagiri (1966) proposes that the jelly does not act as a foothold but that a smaller moiety in the jelly may facilitate adherence of the spermatozoa to the egg surface. The recent work of Barbieri and Villeco (1966) also suggests that a small molecular weight substance present in the jelly is necessary for fertilization.

It can be expected that additional knowledge of the structure and composition of the jelly would permit more enlightened studies on precisely what properties of the jelly are necessary for fertilization. Chemical analyses of amphibian jelly have indicated that the main components of the jelly are fucose, hexosamines, one or more hexoses, and protein (Bolognani *et al.*, 1966; Folkes *et al.*, 1950; Hiyama, 1949a, b, c; Kusa and Ozu, 1961; Lee, 1967; Masamune and Yosizawa, 1953; Masamune *et al.*, 1951; Minganti, 1955; Minganti and D'Anna, 1957, 1958; Schulz and Becker, 1935). However, not all of the above investigations dealt with each of these components and in every case all the jelly layers surrounding the egg were analyzed together; no attention was given to the possible differences among layers. That these differences do exist has been shown by histochemical studies on oviducts and eggs (Ghiara, 1960; Humphries, 1966; Humphries and Hughes, 1959; Kambara, 1956a, b, 1957; Kelly, 1954; Salthe, 1963; Shaver, 1966).

The present investigation was undertaken to study the deposition, structure, and composition of the egg jelly of the South African clawed toad, *Xenopus laevis* (Daudin). Since amphibian jelly is deposited in discrete layers, it was considered of prime importance in this study to analyze these layers separately.

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Histochemical methods, which are useful for this purpose, have been employed extensively in the present work. In addition, it appeared that it would be possible with this species to separate the jelly layers in small quantities for chemical analyses. To the author's knowledge, such chemical analyses of individual jelly layers have not been reported for any amphibian.

MATERIALS AND METHODS

Ovulation of *Xenopus laevis* was induced by an injection of 500 units of chorionic gonadotropin (Antuitrin-S, Parke, Davis).

Cytochemical methods

Oviducts containing eggs were removed from a pithed animal and cut into small segments for fixation. Uterine eggs and deposited eggs were also used. The material was fixed with 10% neutral formalin, 10% neutral formalin containing 0.01 *M* cetylpyridinium chloride (CPC) (Conklin, 1963) or by freeze-substitution (Humphries, 1966). All material was cleared in benzene and embedded in Tissuemat. For most of the staining procedures, serial sections of 10 μ thickness were cut and placed alternately on each of 3 slides.

Carbohydrates. The periodic acid-Schiff reaction was used with and without prior treatment with malt diastase. Both native and boiled enzyme were used in a concentration of 1% in distilled water for 2 hours at room temperature. Toluidine blue was employed in a concentration of 0.1% in distilled water for 1–2 hours. Coriphosphine O (Gurr) was used in a 0.01% solution in distilled water for 15 minutes and sections were examined in distilled water (Humphries, 1966). Detection of fluorescence in coriphosphine-stained material was by means of a Leitz SM microscope using an HBO 200-watt mercury vapor lamp with Leitz exciter filter UV UG1. Alcian blue was employed routinely as a 0.1% solution in 3% acetic acid (pH 2.4–2.6) or was used in increasing concentration of standardized $MgCl_2$ according to the method of Scott and Dorling (1965).

Bovine testicular hyaluronidase (Sigma Chemical Co.) was used in a concentration of 60 USP units/ml. in 0.1 *M* phosphate buffer at pH 6.0. Sections were incubated for 8 hours at 37° C. Cartilage controls were used. Sialidase (neuraminidase, *Vibrio cholerae*, Calbiochem, 500 units/ml.) was used according to the method of Spicer and Warren (1960). Enzyme-treated sections were stained with toluidine blue or alcian blue.

Proteins. Staining was done according to the bromsulfalein method of Silverman and Glick (1966) and the ninhydrin-Schiff procedure of Yasuma and Itchikawa (1953). The dimethylaminobenzaldehyde (DMAB)-nitrite method (Adams, 1957) for tryptophan was also employed. For the detection of sulfhydryl groups the mercury orange method of Bennett and Watts (1958) was used with and without prior treatment with thioglycolate to reduce disulfides (Barka and Anderson, 1963).

Enzymatic treatment of live, jelly-covered eggs

Eggs were stripped from the female and placed immediately into the various solutions. Treatments were carried out at room temperature in native and boiled

enzyme solutions and in solvent controls. Papain (2 × crystallized, Sigma Chemical Co.) was used in a concentration of 0.1 mg./ml. in a 0.1 N phosphate buffer (pH 6.3) containing 5 m*M* cysteine-HCL and 5 m*M* EDTA. Pepsin (N. F., Merck and Co.) was employed in a concentration of 1 mg./ml. in 0.01 N HCl. Hyaluronidase was prepared as described above. All observations were made on the live eggs.

Chemical methods

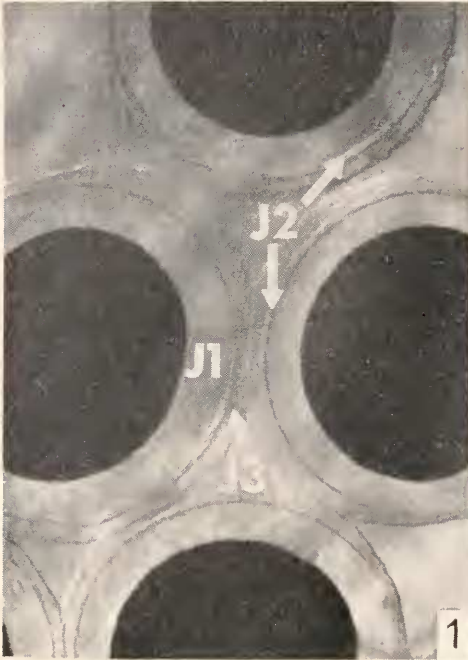
For removal of the jelly, approximately 50 eggs at a time were stripped from a female into distilled water. The jelly layers were removed separately with sharpened watchmaker forceps, placed immediately in vials kept in an ice-salt bath at −10° C. to 0° C. and stored in the frozen state. The jelly coats from the eggs of several females were later pooled and lyophilized.

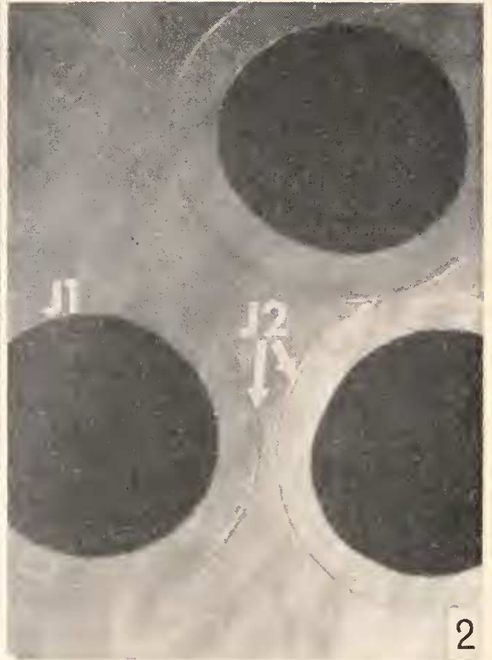
Nitrogen. Nitrogen content of the jelly was determined by the Nessler method (Lang, 1958) using two different procedures. (1) Equal numbers of coelomic and uterine eggs from the same female were assayed and nitrogen content of the jelly was taken as the difference between the two values. In each case additional eggs from the same female were dried at 100° C. to determine weight. (2) One-mg. samples of lyophilized jelly coats were assayed to determine the distribution of nitrogen among the layers.

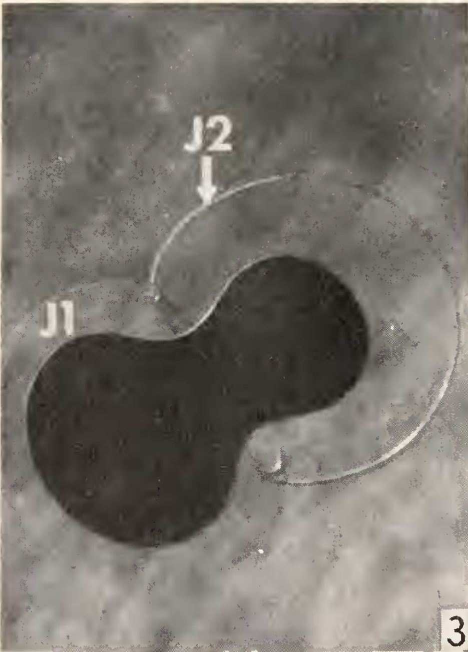
Uronic acids. The method of Galambos (1967) was employed. One-mg. samples of each of the separate, lyophilized jelly coats were dissolved in 0.2 ml. of 1 N NaOH, diluted to 2 ml. with distilled water, and 0.8-ml. portions were used for the Galambos procedure. Chondroitin sulfate (20–400 μg., Nutritional Biochemical Corp.) and glucuronic acid (4–80 μg., crystalline, Sigma Chemical Co.) were prepared in 0.1 N NaOH and used as standards.

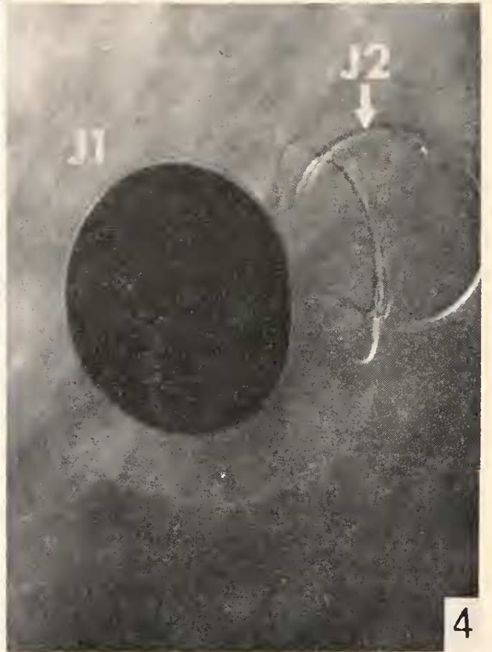
Sialic acid. Total sialic acid was determined by the thiobarbituric acid assay (Warren, 1959, 1960). To study the distribution of sialic acid among the jelly layers, assays were performed on 1–2 mg. of the separate, lyophilized jelly coats. N-acetyl-neuraminic acid (synthetic, Type IV, Sigma Chemical Co.), in concentrations from 0.01 μmole to 0.05 μmole, was used as the standard. Readings were made on a Zeiss spectrophotometer in 3-ml. cuvettes (1-cm. light path).

Paper chromatography. The method of Caldwell and Pigman (1965) adapted from Masamune and Yosizawa (1953) was used to identify sugars present in the separate jelly layers. Approximately 2 mg. of each coat were hydrolyzed in 1–2 ml. 2 N H_2SO_4 in capped tubes in a boiling water bath for 5 hours. The solutions were then brought to approximately pH 6.0 by adding saturated $BaOH_2$ and, after centrifugation, the supernatants were evaporated to dryness at room temperature with a stream of cool air. The sugars were redissolved in 2–4 drops of distilled water and 8–12 μl. were used to spot each chromatogram. Reference sugars (1 mg./ml.), placed on the same paper, included D-glucose, D-glucosamine-HCL, N-acetyl-D-glucosamine, D-galactose, D-galactosamine-HCl, N-acetyl-D-galactosamine, D-mannose, D-mannosamine-HCl, N-acetyl-D-mannosamine and D-fucose (Sigma Chemical Co.). Descending chromatography was carried out in n-butanol-pyridine-water (5:2:2 v/v) on 18.5 × 22 inch Whatman No. 1 chromatography paper in an equilibrated chromatography chamber. The solvent was allowed to drip off the paper and the total time ranged from 40









FIGURES 1–4.

to 62 hours. Reducing sugars were detected by the method of Trevelyan *et al.* (1950) and amino-sugars by an adaptation of the Elson-Morgan reaction for hexosamines (Smith, 1960).

Results

When a uterine egg is placed in tap water the three transparent jelly coats can be observed easily (Fig. 1). The inner layer of jelly (J1) is approximately 0.2 mm. wide and has a gelatinous consistency. The outermost layer (J3) is about 0.1 mm. wide and because it becomes quite sticky in tap water the deposited eggs tend to be held together in masses. Between these two layers is another very thin layer (J2), approximately 0.02 mm. wide, which appears membranous and fairly tough. With forceps, J3 may be removed easily. If J2 is then punctured with the tip of the forceps, the egg, still surrounded by the inner layer (J1), can be pushed out (Figs. 2, 3, 4).

The jelly layers are deposited as the eggs pass through the long, coiled oviduct. As the eggs travel from the coelom into the oviduct they are collected in a thin-walled ostial region. From here they pass singly into the first secreting region where J1 is deposited around the egg. This section of the oviduct is fairly thick-walled and lined with jelly-secreting glands. Approximately half-way down the oviduct the wall becomes even thicker and the glands in this second secreting region can be distinguished histochemically from those in the anterior half of the oviduct. J2 and J3 are formed around the egg as it travels through this region. The eggs then pass into the thin-walled sac-like uterus where they accumulate before egg deposition begins.

Cytochemical observations

The picture observed in the live jelly-covered egg was best retained when the material was fixed by freeze-substitution. Severe shrinkage of the jelly occurred when 10% formalin or 10% formalin containing CPC was used. Since the histochemical reactions were essentially the same after these three fixation methods the material to be described below is that fixed by freeze-substitution unless indicated otherwise.

Both secreting regions of the oviduct and all jelly layers were distinctly PAS-positive. The second secreting region and J2 and J3 reacted more intensely than did the first secreting region and J1. Diastase did not affect the PAS reactivity of the jelly or the oviducal glands but did remove essentially all positivity from the egg. Toluidine blue-stained material in water showed an intense pink to purple metachromasia only in J1 and the glands of the first secreting region. After

All figures: Live *Xenopus laevis* eggs stripped from a female and immersed in tap water. × 30.

FIGURE 1. All three jelly layers are present.

FIGURE 2. J3 has been removed with forceps.

FIGURE 3. J3 has been removed, J2 has been punctured with forceps, and the egg still surrounded by J1 has been pushed partially out of J2.

FIGURE 4. Same as Figure 3, but egg surrounded by J1 has been pushed completely out of J2. J2 remains on outer edge of J1.

alcohol dehydration of formalin-CPC-fixed material, metachromasia of J1 and the first secreting region was retained although diminished considerably in intensity. In material fixed by freeze-substitution, alcohol dehydration usually abolished any metachromasia. With coriphosphine O, J1 and the glands which secrete it gave a bright orange-red fluorescence. J2, J3, and the glands of the second secreting region were unstained or were only a dull orange. With alcian blue at pH 2.4, J1, and the glands of the first secreting region stained bright blue. The egg, J2, J3, and the second secreting region of the oviduct were unstained.

When material was stained with alcian blue at pH 5.8 in increasing concentrations of $MgCl_2$, J1 and the glands in the first secreting region of the oviduct continued to stain until the $MgCl_2$ concentration exceeded 0.5 *M*–0.7 *M*. Glands of the second secreting region and J2 and J3 did not stain in concentrations above 0.3 *M* $MgCl_2$. Magnesium chloride in excess of 0.2 *M* prevented the egg from staining. Rat rib cartilage staining was abolished in concentrations of $MgCl_2$ above 0.9 *M*.

When any of the carbohydrate stains were used on formalin-CPC-fixed material, the inner portion of J1 gave a more positive reaction than the outer portion of J1. With freeze-substitution J1 appeared essentially homogeneous throughout.

After treatment of sections with hyaluronidase or sialidase, there was no noticeable change in toluidine blue or alcian blue (pH 2.4) staining of the eggs, jelly coats, or oviducal glands.

All four methods employed for the detection of proteins gave the same general staining pattern. With ninhydrin-Schiff the egg was bright magenta. The glands in the first secreting region were essentially unstained, and J1 was only a very pale pink in some sections. J2, J3, and the second secreting region gave a consistent positive reaction although the color was only a pale pink. Somewhat brighter staining was observed after formalin-CPC fixation. Bromsulphalein stained the egg deep purplish-blue. J1 and the first secreting region remained unstained. J2, J3, and the second secreting region were very pale blue. With the DMAB-nitrite method for tryptophan the egg was a positive blue. J1 and the glands which secrete it had only occasional traces of a very pale blue. J3 and the second secreting region were a consistent pale blue. J2 was a deeper blue. When mercury orange was used without prior reduction of disulfides, all three jelly layers and the glands of both secreting regions were negative for sulfhydryl groups. Only the egg stained orange. After thioglycolate treatment, the color in the egg increased and J1 and the first secreting region were pale orange. J2, J3, and the second secreting region were a brighter orange.

Enzymatic treatment of live eggs

After about two hours in papain, J3 had disappeared and J1 had begun to liquefy within the intact J2 layer. After an additional two hours all jelly had been removed. Pepsin appeared to cause dissolution of J3 only. To test further whether J1 and J2 were affected by the enzyme, eggs with only J1 and also separate J2 coats were placed in the pepsin solution. In native enzyme and control solutions J1 shrank but remained around the egg. Separate J2 coats also shrank but, after as long as eight hours, no dissolution of J2 was noticed. Hyaluronidase had no apparent effect on the jelly.

Chemical analyses

When the jelly coats were removed manually from the egg for chemical analyses, it was observed that J3 could be removed without disturbing the other layers and so was probably free of other material. J2 contained a small amount of material from J1 and perhaps also a lesser amount from J3. J1 was thought to be free of contamination from J2 and J3 but, since not all of J1 could be removed without cytolyzing the egg, the innermost part of J1 was not included in the following analyses of the jelly.

Table I gives the values for nitrogen content of: (1) the entire jelly of the eggs of three females (designated A, B, and C); and (2) lyophilized samples of the three separate jelly coats collected from the eggs of several females.

TABLE I

Nitrogen content of eggs and jelly layers

Type of test*	Sample	Mean μg. N/egg (Std. error)	N as % of dry wt. of jelly
(1) Eggs from:			
Female A	coelomic eggs	24.62(±0.40)	6.9%
	uterine eggs	27.05(±0.64)	
Female B	coelomic eggs	46.91(±0.61)	7.9%
	uterine eggs	51.63(±0.22)	
Female C	coelomic eggs	34.63(±0.27)	12.8%
	uterine eggs	38.34(±0.74)	
(2) Lyophilized jelly coats:	J1		7.1%
	J1		7.9%
	J2		8.1%
	J2		6.0%
	J3		6.8%
	J3		7.4%
	J3		6.8%

* See explanation in "Materials and Methods."

Table II gives the results of sialic acid determinations on separate, lyophilized jelly coats. On a dry weight basis J1 appears lowest in sialic acid with 0.14%. J2 has the highest average value, 0.40%, but substantial variations were found in these samples. J3 consistently contained 0.37%. Hydrolysis for three hours did not result in higher sialic acid values but, in fact, lower values were often obtained.

The test for uronic acid content of the three separate jelly layers was negative.

Chromatography

For each jelly layer three separate chromatography runs were made. Duplicate sheets were included in each run so that the two detection reagents could be used.

Table II

Sialic acid content of the separate jelly layers

Sample	Number of samples†	Hydrolysis time	Sialic acid μg./mg. dry wt.*	Sialic acid % of dry wt.
J1	3	1 hour	1.39(1.17–1.51)	0.14%
J2	3	1 hour	4.02(2.30–6.00)	0.40%
J3	3	1 hour	3.71	0.37%
J1	1	3 hours	0.62	0.06%
J3	1	3 hours	2.78	0.28%

† Number of samples used to get average values listed.

* Equation 2 of Warren (1959) was used for all calculations and 309, the molecular weight of N-acetylneuraminic acid, was used in order to express concentrations in micrograms.

In Table III are listed the sugars present in each jelly coat as found by comparing the $R_{glucose}$ values of the unknown sugars with those of the standards and by comparing the colors obtained after using the Elson-Morgan reagent.

Discussion

The only three descriptions available for the jelly of *Xenopus laevis* eggs are brief and conflicting. Deuchar (1966) reports two jelly coats, Salthe (1963) suggests five coats, and Ghiara (1960) observed three: a thin inner layer, a wide middle layer, and a thin outer coat. He noted metachromasia in the two inner layers after staining live eggs with toluidine blue. The present report presents evidence that there are three coats: a wide, gelatinous inner coat, a very narrow, membranous middle layer, and a fairly wide outer coat.

The inner layer of jelly appears to contain neutral and acidic polysaccharides and possibly protein. Toluidine blue metachromasia, intense alcian blue staining, and a bright orange-red fluorescence with coriphosphine O strongly suggest the presence of some acid groups whose identity remains unknown. The tests for uronic acids were negative. Similar negative results have been reported for the other amphibians investigated (Bolognani *et al.*, 1966; Folkes *et al.*, 1950; Hiyama, 1949a). It is doubtful that the intense alcian blue (pH 2.4) staining of J1 is due to carboxyl groups because at pH 2.6 or lower, carboxyl group staining may be masked as a result of salt links with proteins (Scott and Dorling, 1965). Using alcian blue at pH 5.8 in increasing concentrations of $MgCL_2$, Scott and Dorling (1965) found that carboxyl group staining can be abolished at a critical electrolyte

Table III

Sugars identified in each jelly layer

J1	Fucose, galactose, glucosamine, galactosamine, glucose?*
J2	Fucose, galactose, glucosamine, galactosamine
J3	Fucose, galactose? glucosamine, galactosamine, mannose? mannosamine?

* Question marks in the table indicate that a spot with an R_g value corresponding to that particular known sugar was found in only one of the three chromatography runs.

concentration (CEC) of approximately 0.3 *M* $MgCl_2$ and sulfate at 1.0 *M* or greater; a mixture of the two, such as found in heparin, has an intermediate CEC. The CEC for sialic acid-containing structures is approximately 0.2 *M* $MgCl_2$ (Quintarelli and Dellovo, 1965). Since the CEC of layer J1 is about 0.7 *M*, staining of this layer cannot be accounted for solely by sialic acid. Also, the toluidine blue and the alcian blue stainings were not affected by sialidase treatment. The above evidence and also the persistence of some toluidine blue metachromasia after alcohol dehydration suggest the presence of sulfate in J1 and the glands of the region which secrete it. Past evidence (Minganti, 1955; Lee, 1967) has indicated that amphibian egg jelly has no sulfate. However, other biochemical reports have suggested that sulfate may be present (Minganti and D'Anna, 1958; Bolognani *et al.*, 1966) and, in previous histochemical investigations, several species of amphibians have been found to exhibit metachromasia in the jelly and/or oviducts (Ghiara, 1960; Humphries, 1966; Humphries and Hughes, 1959; Kambara, 1957; Katagiri, 1963a; Kelly, 1954; Shaver, 1966). In no case has the substance responsible for this metachromasia been identified.

The source of the J2 layer is still in question. Although both J2 and J3 appear around the egg in the second secreting region of the oviduct, there has been no way to subdivide further this part of the oviduct and locate precisely the site of deposition of J2. The staining reactions of J2 resemble those of J3 although the reactions for neutral polysaccharides and proteins in J2 are more intense. This may be due in part to the construction of J2, which in sections appears to consist of rather tightly packed fibers arranged parallel to the egg. J2 is not metachromatic and is alcian blue negative at pH 2.4. The alcian blue staining at pH 5.8 is similar to that of J3 and is probably due to amino acids and/or sialic acids, not to uronic acids. Unlike J3, and similar to J1, J2 does not dissolve upon treatment with pepsin. In live material its consistency is quite different from either of the other two layers. It is fairly tough and membranous and not at all sticky as is J3; when J3 is removed, the eggs are no longer held together in masses. Possibly, J2 as such is not deposited by the oviduct but is formed when J3 is deposited on J1. Perhaps some complexing of the components of J1 and J3 occurs, which might change the enzyme susceptibility and staining characteristics of these components. Unless some material is found which is unique to J2 it will be difficult to establish that it is a truly distinct layer and that it is deposited as such by a particular region of the oviduct. There may be a similar situation in the case of *Rana pipiens,* where, although the egg has three jelly layers, the middle part of the oviduct appears immunologically to be an area of overlap between the upper and lower regions (Barch and Shaver, 1963). Katagiri (1963a) describes for the eggs of *Hyla arborea japonica* a "jelly membrane" between the inner and the outer two layers of jelly; as spermatozoa penetrate the jelly they come to a standstill momentarily at this "jelly membrane." Katagiri also suggests that changes in the "jelly membrane" are a prime cause of the decrease in fertilizability of the eggs which have been standing in tap water. Tchou and Wang (1956) noticed that when the eggs of *Bufo bufo asiaticus* remain in water for some time a distinct membrane forms between the outer and inner jelly layers which may act as an obstacle to sperm penetration.

J3 appears to be composed predominantly of neutral polysaccharides and some

protein. The presence of carboxyl groups is indicated and, since there is no uronic acid, these groups could be due to amino acids and/or to the small amount of sialic acid that is present. The actual values obtained for sialic acid are probably much too low because fucose, which has been found in all jelly layers, causes abnormally low readings with the method used (Warren, 1959). Recently, sialic acid has also been found in the oviduct and/or egg jelly of other amphibians (Humphries and Workman, 1966; Humphries *et al.*, 1968; Bolognani *et al.*, 1966; Lee, 1967). Soupart and Noyes (1964) demonstrated histochemically the presence of sialic acid in the zona pellucida of the ova of several mammals, and the possible significance of the compound was investigated by Soupart and Clewe (1965) who treated rabbit ova with neuraminidase and found deformation of the zona pellucida and a definite inhibition of sperm penetration.

In view of the differences observed histochemically in the jelly layers of *Xenopus* eggs it is interesting that, qualitatively, the sugar components of the three layers are nearly identical. From the preliminary work reported here, all layers appear to contain fucose, glucosamine, and galactosamine. Galactose is present in J1 and J2 and possibly in J3. In addition, J3 may contain mannose or mannosamine. The sugars may be joined together in polysaccharides and/or may be attached to proteins. It appears from the histochemical reactions that protein is present in all three jelly layers although these tests are not completely satisfactory since they do not actually indicate protein but are specific for certain reactive sites or for particular amino acids. However, proteolytic digestion of the jelly does suggest that the amino acids are linked together in proteins and, furthermore, the enzymatic treatment has served to demonstrate that some differences do exist among the proteins of the three jelly layers.

In conclusion, it is obvious that the jelly capsule is composed of discrete layers. The separate layers appear to differ chemically and physically and as a result they may have quite different functions. To pool all layers for chemical analyses or for studying fertilization may give misleading results. Information about the macromolecules of the jelly layers is now needed.

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Summary

1. The deposition, structure, and composition of the jelly surrounding the egg of *Xenopus laevis* were studied cytochemically and biochemically. During its passage through the oviduct, the egg is invested with three layers of jelly designated J1, J2, and J3, from innermost to outermost. Particular emphasis was placed on analyzing the layers separately.

2. All layers gave positive histochemical tests for neutral polysaccharides. J2 and J3 stained the most intensely. The sugars present in the polysaccharides of each layer were identified chromatographically. J1, J2, and J3 all contain fucose, glucosamine, and galactosamine. Galactose is present in J1 and J2 and possibly in J3. J3 may also contain mannose or mannosamine.

3. On the basis of the histochemical tests it is suggested that J1 contains sulfate. The weak positive reactions for acid polysaccharides in J2 and J3 indicate the presence of carboxyl groups only. Since no uronic acid was found in the jelly it is proposed that the carboxyl groups are part of amino acids and/or sialic acid which was found in small amounts in all layers.

4. The nitrogen values for the three layers are similar. All layers gave positive histochemical tests for protein although the reactions in J1 were weak and irregular. Treatment of live eggs with proteolytic enzymes showed that all jelly layers do contain some protein as an important structural component and that the protein components of the three layers are not identical.

5. It is suggested that future studies should be concerned with characterizing the macromolecules of the jelly and that, for all investigations of the structure and function of the jelly, the differences among layers should be taken into account.

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